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# Methylation of the chicken vitellogenin gene: influence of estradiol administration

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## ABSTRACT

The degree of methylation of the chicken vitellogenin gene has been investigated. Upon induction by administration of estradiol to a rooster, methyl groups at specific sites near the 5'-end of the gene are eliminated. The process of demethylation is slower than the activation of the gene. Demethylation is therefore probably not a prerequisite to gene transcription. At least two other sites in the coding region of the gene are methylated in the liver of estrogenized roosters, but not in the liver of a laying hen, where the gene is naturally active.

## INTRODUCTION

5-Methylcytosine is the only modified base occurring in DNA of vertebrates. It is found primarily, but not exclusively, in the doublet CpG (1-4). Several possible roles of DNA methylation in eukaryotes have been proposed, including regulation of gene expression, chromosome inactivation, and regulation of DNA replication. Occurrence and possible functions have been reviewed (5,6).

Several restriction enzymes have been found useful tools for the study of DNA methylation (7). For instance, *Hpa*II and *Msp*I both recognize the sequence CCGG, but *Hpa*II will only cleave if the internal cytosine is not methylated, whereas *Msp*I will only cleave if the external cytosine is unmodified (8,9). Thus digestion of genomic DNAs with these restriction enzymes and comparison of the fragments produced, allows the detection of methylation at specific sites.

The results of experiments of this type have yielded evidence for an inverse correlation between methylation and transcriptional activity of many genes, amongst which globin genes (10-12), viral genes in virus-transformed cells (13,14), and the estradiol-controlled genes coding for ovalbumin, conalbumin and ovomucoid (15). Furthermore, certain genes are activated in cultured cells which are treated with the hypomethylating cytidine analog 5-azacytidine (16,17). Some authors have suggested a direct relationship

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between chromatin structure -as monitored by nuclease digestion- and methylation (12,18,19).

Our experimental system, the induction of vitellogenin synthesis by estradiol in the chicken liver, is an attractive one for this kind of studies. A major advantage is that even before activation of the genes, the target tissue is available for study. In contrast to, for instance, the oviduct system, induction is not dependent on cell proliferation. The vitellogenin gene can simply be activated by administration of 17-β estradiol to a rooster; after three to four days, the protein synthesis and mRNA concentration have reached their maximum level (20,21). We have compared the methylation status of the gene before and after induction. In this paper we demonstrate that demethylation of specific sites is correlated with gene activity.

## MATERIALS AND METHODS

### Animals

White Leghorn roosters and hens were obtained from Poultry Hatchery Van der Sterren, Venray, The Netherlands. Roosters were injected subcutaneously with estradiol (50 mg/kg body weight). The animals were 6-10 weeks old unless otherwise stated.

### DNA preparations

To remove erythrocytes, livers were perfused in situ for approximately 5 minutes with 100-200 ml Krebs-Ringer buffer (4.8 mM KCl, 1.2 mM $KH_2PO_4$, 1.2 mM $MgSO_4$, 121 mM NaCl, 24 mM $NaHCO_3$) which was saturated with 95% $O_2$, 5% $CO_2$ and kept at 37°C. To isolate parenchymal cells, perfusion was then continued with a Dispase II (Boehringer Mannheim) solution of 1 Unit per ml Krebs-Ringer buffer. Then the liver was removed and pressed through a coarse nylon sieve and subsequently the disaggregating tissue was filtered through 4 layers of 100-mesh nylon screening. The cells were centrifuged in a HOMEF clinical centrifuge for a few seconds at 1500 rpm. The pellet was resuspended in Krebs-Ringer buffer with a syringe, and the cells were recentrifuged three times. The parenchymal cell preparations were at this stage at least 95% pure, as was concluded from inspection with the light microscope. DNA was purified from these cells according to Blin & Stafford (22). DNA preparation from other tissues was according to Ref. 23. White Leghorn sperm was obtained from the Spelderholt Institute for Poultry Research, Beekbergen, The Netherlands. Embryo DNA was purified (according to Ref. 22) from isolated nuclei that originated from the pooled livers of 25 male and female 19-day old embryos.

Isolation of specific DNA fragments from agarose gels was according to Tabak and Flavell (24).

Restriction and blotting analysis of genomic DNA

Restriction enzymes were purchased from New England Biolabs except for EcoRI and BamHI which were isolated according to Greene et al. (25). Incubation conditions were as described by the manufacturers and the authors of Ref. 25, respectively. HpaII and MspI were used at 5-15 fold excess and incubated for 3-10 hours. Completeness of the digestion was checked by parallel digestion of genomic DNA mixed with λ-DNA. Hybridization conditions of Southern blots (26) were essentially as described in Ref. 27.

Scanning of autoradiograms

Autoradiograms were scanned using an Optronics Photoscan. The density profile was visualized with a Tectronics Storage Scope, and the peaks were photographed and subsequently quantitated using a Hewlett-Packard 9864A digitizer and 9820A calculator.

## RESULTS

Approach

The availability of cloned fragments containing different portions of the vitellogenin gene region (28) enabled us to study the methylation in specific sections of the gene in genomic DNA from different tissues or chickens. Fig. 1 shows the position of the clones used, with respect to the vitellogenin gene. It also indicates the location of relevant restriction sites, namely the HpaII sites, three of which are numbered HpaII-0, HpaII-1, and HpaII-2, and a HhaI site. For the purpose of obtaining a picture of the

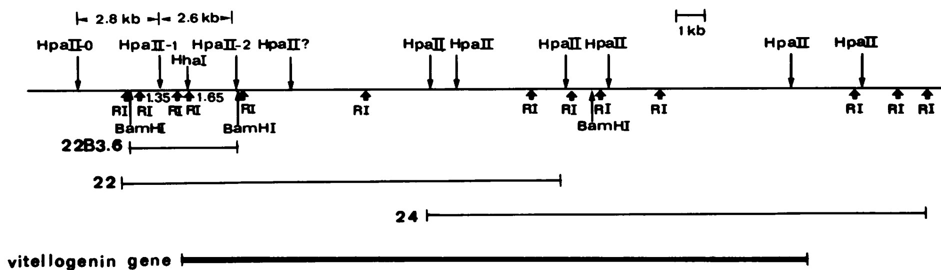


Fig. 1. Map of the chicken DNA region containing the vitellogenin gene. A simplified restriction map of the gene region is shown at top. RI= EcoRI; 1350 and 1650 refer to lenghts in base pairs of two EcoRI fragments that were used as probes in some experiments. The positions of the λ Charon 4A recombinant clones 22 and 24 (Ref. 28) and of the plasmid subclone 22B3.6 are indicated underneath the map.

overall methylation state of the gene, we hybridized HpaII digests of DNAs from various tissues with clones 22 or 24. These lambdoid clones roughly contain the 5'- and 3'- halves of the gene, respectively. Subsequently, we used isolated restriction fragments to study the extent of methylation of some individual sites near the 5'- terminus of the gene. One of these fragments, a 3.6-kb BamHI fragment was cloned in pBR322; this subclone is referred to as 22B3.6.

Hybridizations with λ clones covering the entire vitellogenin gene

Fig. 2 shows the results of Southern blot hybridization of HpaII-digested genomic DNAs with labeled clone 22 DNA. Fragments of relatively high molecular weight are detected in digests of DNA from livers of embryos and non-stimulated roosters (lanes 1 and 2), indicating a high extent of methylation of most sites in the vitellogenin gene region. With the 5'-specific probe clone 22, a shift to fragments of higher mobility is observed in digests of parenchymal cell DNA from roosters at 3 days (Fig. 2, lane 3) or 32 days (Fig. 2, lane 4) after estradiol treatment. Two fragments of approximately 9.3 and 9.4 kb appear that were absent in the HpaII digest of DNA from the non-stimulated rooster,

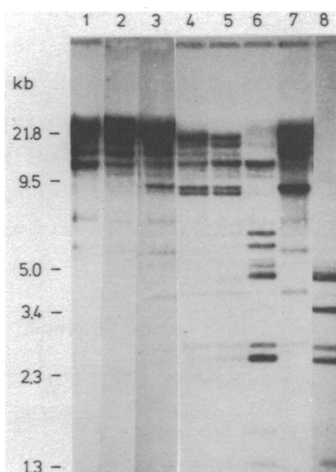


Fig. 2. Overall methylation state of the vitellogenin gene.
Genomic DNAs (appr. 10 μg) were digested with HpaII, electrophoresed in 0.7% agarose gels, transferred to nitrocellulose and hybridized with [ $^{32}$P]-labeled clone-22 DNA. The DNAs originated from the livers of : embryos (lane 1), a control rooster (lane 2), roosters at 3 days (lane 3) and 32 days (lane 4) after estradiol treatment, a rooster after repeated -at 32, 18 and 4 days before it was killed- estradiol treatment (lane 5), a laying hen (lane 6); from the oviduct of a laying hen (lane 7). Lane 8 contains MspI-digested liver DNA of an estrogen-treated rooster.

and even smaller bands are vaguely visible. The demethylation is much more pronounced in the DNA from laying hen liver (Fig. 2, lane 6). In this naturally active gene, an extensive demethylation is evidenced by the appearance of strong bands corresponding to fragments of 2.6 and 2.8 kb and other fragments of relatively high mobility. The oviduct pattern (lane 7) contrasts with, for instance, laying hen liver DNA (lane 6); these DNA preparations originate from the same animal.

Hybridizations with clone-24 DNA, corresponding to the 3'-half of the gene, did not show a clear demethylation in this part of the gene in estrogenized roosters; but some demethylation occurred in the laying hen liver (data not shown).

The results discussed above suggest that some sites in, or surrounding the 5'-half of the gene become demethylated upon estradiol treatment. This tentative conclusion is supported by hybridization with labeled 22B3.6 DNA (Fig. 3). The observation of estradiol-dependent demethylation, similar to the findings with clone 22 as a probe, indicates that the demethylation occurs near the region covered by 22B3.6. The conspicuous appearance of the 2.6-kb and 2.8-kb HpaII-fragments which are bracketed by sites HpaII-0, HpaII-1, and HpaII-2 (see Fig. 1), shows that these sites become strongly

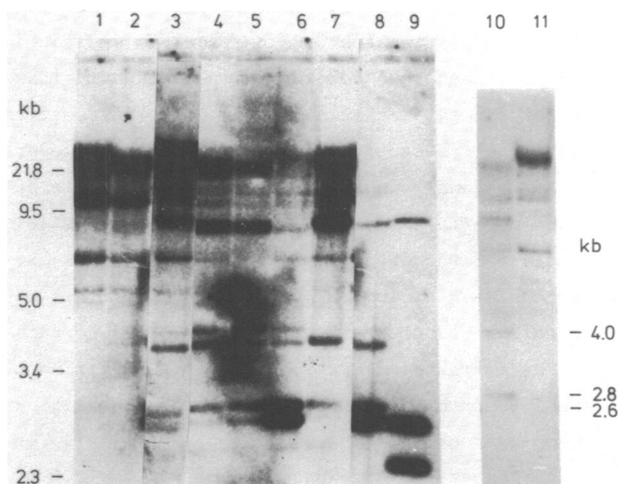


Fig. 3. Methylation around the 5'-end of the vitellogenin gene
Southern blots containing HpaII-digested genomic DNAs were hybridized against labeled 22B3.6 DNA. Lanes 1-8 are layered as in Fig. 2; lane 10 with liver DNA and lane 11 with erythrocyte DNA, both from an 8-month old rooster. Lane 9 contains MspI-digested clone-22 DNA.

demethylated. In HpaII-digested (non-methylated) clone-22 DNA, used as a control (Fig. 3, lane 9) we see the 2.6-kb fragment, while a 1.8-kb fragment appears in lieu of the 2.8-kb fragment, only part of which is present in clone 22.

Note that 32 days after a single estradiol treatment, the liver of a rooster has already ceased to synthesize vitellogenin (20,21). Nevertheless the demethylation effect is more pronounced after 32 days (Figs. 2 and 3, lane 4) than after 3 days (Figs. 2 and 3, lane 3), when the gene is highly active. When a rooster was estrogenized repeatedly, 32 days, 18 days and 4 days before it was killed, the pattern obtained was similar to the pattern from the rooster 32 days after a single treatment (Figs. 2 and 3, lanes 5 and 4). However, in the DNA from the rooster that has been treated repeatedly, the intensity of the 2.6-kb HpaII band has become stronger compared with the intensity of the 2.8-kb band, suggesting an additional demethylation of HpaII-2.

Effect of ageing

As shown above (see Figs. 2 and 3, lanes 5-7), the active vitellogenin gene in an 8-month old laying hen is less methylated than in estrogenized cockerels. We questioned whether the age of the animals influences the methylation of the gene. Therefore, we hybridized labeled 22B3.6 DNA with HpaII-digested liver DNA of an 8-month old rooster. Fig. 3, lane 10 shows the result: a significant higher percentage of the hybridization signal is found in some smaller bands among which the 2.8-kb fragment, indicating the elimination of methyl groups. Thus some demethylation of the vitellogenine gene occurs in the liver of a rooster that has never been treated with estradiol. We assume that this is an effect of ageing because we have never observed a similar demethylation when analysing several control cockerels of the same inbred strain. No ageing effect is visible in erythrocyte DNA (Fig. 3, lane 11).

In 1975, Holliday and Pugh hypothesized (29), that cell-division-coupled demethylation functions as a biological clock and has in this way a role in the process of ageing. It is noteworthy, that there is a certain degree of similarity between the patterns of HpaII fragments hybridizing with 22B3.6 from the DNA of the oviduct of an 8-month old laying hen (Fig. 3, lane 7) and of an 8-month old non-estrogenized rooster (Fig. 3, lane 10). For instance, in both patterns the 2.8-kb band is more pronounced than the 2.6-kb band, and there is a fairly strong band of about 4 kb.

Methylation degree of individual sites

To investigate the methylation degree of HpaII site 1, we hybridized blots of genomic DNAs that were digested with both HpaII and BamHI with nick-

translated 22B3.6 DNA. Methylation of HpaII-2 is not of significance in this experiment, since this site almost coincides with the 3' BamHI site. In this approach, the extent to which the 3.6-kb fragment is cleaved into its 2.4- and 1.2-kb products, is a measure of the degree of methylation of HpaII-1. Alternatively, methylation of the same site was investigated by labeling the 1.35-kb EcoRI fragment containing HpaII-1 (see Fig. 1) and hybridizing it against blots of HpaII x EcoRI-digested DNAs. This experiment (Fig. 4) clearly demonstrates a progressive demethylation process of HpaII-1, after administration of estrogen to a rooster. Not unexpectedly, HpaII-1 is highly resistant to HpaII in erythrocyte and sperm DNA (Fig. 4B, lane 1 and 2). The reduced resistance of HpaII-1 in an 8-month old rooster is confirmed (Fig. 4B, lane 4). Note that we do not detect any difference in extent of methylation of HpaII-1 between estrogenized rooster (32 days after treatment) and a laying hen (lanes 6 and 7). Therefore, the differences between these DNAs, observed in the experiments described earlier (Figs. 2 and 3) must be due to demethylation of

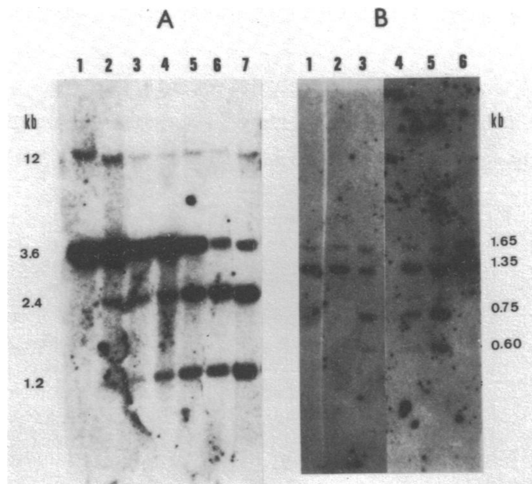


Fig. 4. Methylation of HpaII-1.
Panel A, DNAs were digested with BamHI + HpaII, or with BamHI, electrophoresed, blotted and hybridized with labeled 22B3.6-DNA. Lane 1 contains BamHI-digested erythrocyte DNA. Lanes 2-7 contain BamHI + HpaII-digested liver DNAs from embryos (lane 2), a control rooster (lane 3). roosters at 16 hours (lane 4), 3 days (lane 5) and 32 days (lane 6) after induction, and laying hen (lane 7).
Panel B, DNAs were digested with EcoRI + HpaII, or with EcoRI, electrophoresed, blotted, and hybridized with the 1.35-kb probe. Lanes 1-5 contain EcoRI + HpaII-digested DNAs from erythrocytes (lane 1), sperm (lane 2), oviduct (lane 3), liver of an 8-month old rooster (lane 4), liver of a laying hen (lane 5). Lane 6 contains EcoRI-digested erythrocyte DNA.

other sites. For instance, the higher intensity of the 2.6 and 2.8-kb fragments in the digested laying hen liver DNA, compared to the same fragment in the stimulated rooster, indicates that HpaII-0 and HpaII-2 are stronger demethylated in laying hen liver DNA. In the BamHI digests, we observe a weak signal from a 12-kb fragment of unknown origin, apparently due to a certain degree of similarity to the 3.6-kb fragment.

To monitor the extent of methylation of HpaII-2, we isolated from 22B3.6 a probe, specific for the 1.65-kb EcoRI fragment, and hybridized it against blots of genomic DNAs which had been digested with EcoRI and HpaII (Fig. 5). HpaII should cleave the 1.65-kb EcoRI fragment into 1.3-kb and 0.35 kb fragments if HpaII-2 is unmodified. The only DNA in which HpaII-2 is not highly resistant, originated from laying hen liver (Fig. 5, lane 6). In this DNA, approximately 70 % of the 1.65-kb fragment is cleaved. 'Artificial' gene activation in roosters is apparently not correlated with demethylation of HpaII-2 (lanes 2-5). In the oviduct, HpaII-2 is highly modified.

We have quantified our results by scanning the autoradiograms. A summary of the data, including the results of some additional experiments(not shown) is given in Table I.

Since the 1.35-kb and 1.65-kb EcoRI fragments were not subcloned, but directly extracted from agarose gels, they were not completely pure, which resulted in a slight cross-hybridization by these probes. The extent of this

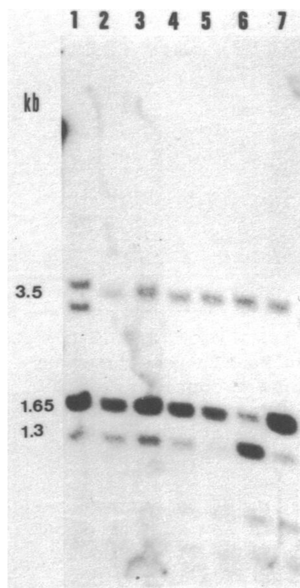


Fig. 5. Methylation of HpaII-2.
Genomic DNAs were digested with EcoRI + HpaII, or with EcoRI, electrophoresed, blotted, and hybridized with a probe specific for the 1.65-kb EcoRI fragment Lane 1 contains EcoRI-digested erythrocyte DNA, lanes 2-6 contain EcoRI + HpaII-digested liver DNAs from a control rooster (lane 2), roosters at 16 hours (lane 3), 3 days (lane 4) and 32 days (lane 5) after induction, and laying hen (lane 6); and oviduct DNA from a laying hen (lane 7).

co-hybridization is demonstrated by hybridization of EcoRI single digests (Fig. 4 lane 6; Fig. 5 lane 1). Furthermore, the 1.3-kb fragment that is cleaved off by HpaII from the 1.65-kb EcoRI fragment, co-migrates with the cross-hybridizing 1.35-kb EcoRI fragment. The necessary corrections for these complications are included in the data of Table I. The fragments larger than 1.65 kb in Fig. 5 are presumably of the same origin as the 12-kb fragment in Fig. 4.

Methylation of an HhaI site in exon 3 of the vitellogenin gene

HhaI recognizes GCGC but will not cleave this sequence if the C in the CpG doublet is methylated. The 3.6-kb BamHI fragment that contains the transcription start site of the vitellogenin gene, contains only one HhaI site, which divides the BamHI fragment in portions of 2.2 and 1.4 kb (K. Kok, unpublished). Sequencing revealed that the HhaI site is located in exon 3, 54 bp from the border with intron 3 (to be published elsewhere). We hybridized labeled 22B3.6 DNA against blots of BamHI + HhaI-digested genomic DNAs. In DNAs from rooster liver, irrespective of whether treated with hormone or not, and in erythrocyte, sperm as well as in oviduct DNA, the HhaI site is highly resistant (>95%). In contrast, in liver DNA from a laying hen, the degree of methylation of the HhaI site is only 40-50% (data not shown). The behaviour of this site is therefore reminiscent of HpaII site 2, which is also located in an exon.

## DISCUSSION

In this paper we describe investigations on a possible role of DNA methylation in the control of the expression of the vitellogenin gene. Although the restriction assay monitors only the methylation degree of a small subset of the potential methylation sites, the results may nevertheless provide some

Table 1. Extent of methylation of HpaII-1 and HpaII-2

| | | % resistance to HpaII | |
|---|---|---|---|
| Animal | Organ | HpaII-1 | HpaII-2 |
| Cockerel, untreated | liver | 90 | 90 |
| Cockerel, 16 h after estradiol treatment | liver | 80 | 90 |
| Cockerel, 72 h after estradiol treatment | liver | 40 | 90 |
| Cockerel, 32 days after estradiol treatment | liver | 20 | 90 |
| Laying hen, 8 months old | liver | 20 | 30 |
| | oviduct | 40 | 90 |
| Rooster, untreated, 8 months old | liver | 70 | 90 |

insight in the correlation between the methylation of the gene and its activity.

We conclude that administration of estrogen to a rooster causes in liver DNA-specific demethylation of at least one site, HpaII-1, near the 5'end of the gene. However, there is no absolute correlation between gene activity and methylation of HpaII-1. Firstly, withdrawal of the hormone, which inactivates the gene does not result in remethylation of this site and, secondly, demethylation may to a lesser extent also occur as a consequence of ageing of the animal. Moreover, demethylation after estradiol administration proceeds slower than the activation of the gene. Thus, demethylation is probably not an early effect of estradiol treatment and should possibly be regarded as a consequence or a side effect of gene activation. However, in our restriction analysis we cannot discriminate between completely and hemi-methylated sites, while it is conceivable that demethylation of a site in only one strand is of biological significance. Although hemi-methylation occurs very infrequently (30), it might be an intermediate stage during activation of the gene. Another remote possibility preventing us from drawing definitely the conclusion that transcription precedes demethylation, is, that only the small fraction of DNA in control rooster liver DNA, that is not methylated at HpaII site 1, is transcribed during the first stage of induction.

At 16 hours after hormone treatment, the methylation degree of HpaII site 1 has decreased only from 90 to 80%. Furthermore, the demethylation continues after the transcription rate of the gene has reached its maximum level at about 72 hours. Repeated estradiol treatments only slightly increase demethylation. It appears that the first contact with the hormone initiates a process that slowly progresses, largely irrespective of additional hormone treatments.

The slowness of the demethylation process may reflect its possible dependence on cell division. Singer et al.(31) proposed that DNA methylation takes place by inhibition of maintenance methylation during replication. This hypothesis implies, that it would take two cell divisions to yield DNA molecules with completely demethylated sites. Estradiol administration to a rooster induces a highly synchronized cell division of 35-40% of the liver cells after 24-30 hours (32). Obviously, this cell division cannot account for the entire demethylation effect, but it may contribute significantly to the decrease from 80 to 40% of HpaII site 1 between 16 and 72 hours. Vitellogenin induction is not dependent on DNA synthesis, in contrast with most systems in which the correlation between DNA methylation and gene activity has been studied. In these systems, induction of a gene requires several rounds of cell division.

It seems likely, that the unmethylated state of the active genes, that is usually found, is generated during these cell divisions, while in our system gene activation precedes cell division, and demethylation.

It is tempting to speculate on a possible relation between the demethylated state of the gene that remains at least 32 days after withdrawal of the hormone, and the so-called memory effect. This phenomenon was discovered by Bergink et al. (20), who showed that after a rooster had been stimulated once with estradiol, it responds more strongly to a second treatment even if the second injection is given several months after the first one. The demethylated state of the gene, which is conserved in the cell and inherited after cell multiplication, may be the persistent factor that boosts vitellogenin synthesis in the secondary stimulation.

Other sites respond differently to the hormone: HpaII-2 is strongly demethylated in laying hen liver, but remains resistant in estradiol-treated roosters after a single estradiol treatment. For a HhaI site, like HpaII-2 situated in an exon, similar results were obtained. Because HpaII-2 becomes slightly demethylated after repeated estradiol treatments (Figs.2 and 3) we currently investigate whether prolonged presence of estradiol is required to demethylate this site.

Williams et al. (33) have reported that the chicken liver contains about 70% parenchymal cells. Inspection with the light microscope of our cell preparations showed that they do not contain more than 5% non-parenchymal cells. The residual methylation of HpaII-1 in, for instance, the laying hen liver DNA can therefore not, or not only, be attributed to such contamination. Kuo et al. (18) demonstrated, that a similar residual methylation of sites in the ovalbumin and conalbumin genes in oviduct DNA could be attributed to a fraction of the chromatin in which these genes are DNAseI resistant, and therefore probably not actively transcribed.

MspI resistance of the CCGG-site, which is attributed to methylation of the external C (4,9) shows considerable variation among the livers of roosters (data not shown). We could not infer any correlation between MspI resistance and activity of the vitellogenin gene. Furthermore, DNAs from the livers of individual control roosters already showed different MspI patterns.

During the course of our work, a report of Wilks et al. (34) on the methylation of the chicken vitellogenin gene was published. These workers too, suggest that there is a specific demethylation of the 5'-region of the gene.

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